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Demonstration of Glutamate Dehydrogenase in Azotobacter Vinelandii

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DEMONSTRATION OF GLUTAMATE DEHYDROGENASE IN *AZOTOBACTER VINELANDII*

BY

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AZOTOBACTER VINELANDII

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor Date

Head, Department of Bacteriology Date

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INTRODUCTION

In the early part of this century it was observed that certain microorganisms could grow in the absence of fixed nitrogen. Since those early observations there has been an intensive effort to unravel the mystery behind the physiology of nitrogen fixation. It was not until development of the use of heavy isotopes that we began to learn the mechanism of biological nitrogen fixation.

When Azotobacter vinelandii was exposed to N_2 for a short time and the nitrogen containing compounds were analyzed for labeled compounds, the highest amount of label was found in NH_4^+. We believe that the ammonia may not be released into the cytoplasm but that a keto acid may receive the amino group directly from the enzyme surface. If this is true, then there is a possibility that glutamate dehydrogenase would not be present in the cell when it is fixing nitrogen. Glutamate dehydrogenase (E.C. 1.4.1.2. L-glutamate: NAD oxidoreductase deaminating) catalyzes one of the principal reactions by which cells assimilate NH_4^+.

It was the object of this study to determine if glutamate dehydrogenase (GDH) was present in A. vinelandii when it was fixing nitrogen. If it is assumed that GDH is the principal means of assimilation of NH_4^+ by A. vinelandii, it should be present when cells are fixing nitrogen if NH_4^+ is an intermediate in nitrogen fixation. If GDH were found in cells fixing nitrogen

this would not be proof of its involvement in the process. However, if it were shown to be absent during nitrogen fixation and present during NH_4^+ assimilation, one can assume that it plays no role in nitrogen fixation. For this to be true GDH must be an inducible enzyme.

REVIEW OF LITERATURE

Since Beijerinck's (3) first description of Azotobacter and its ability to utilize atmospheric nitrogen, there has been a concerted effort to find out how this very important biological mechanism functions. During the first half of this century many studies, using cell-free extract, were conducted in an attempt to learn more about the mechanism of nitrogen fixation. However, it was not until 1965 when Bulen et al. (5), using extracts of A. vinelandii, demonstrated cell-free nitrogen fixation. Then productive studies of the mechanism of nitrogen fixation could begin. Burris (7) stated that Bach et al. obtained cell-free fixation of N_2 as early as 1934 but their results were not reproducible. In 1960, Nicholas and Fisher (26) reported that they had achieved cell-free fixation earlier but later stated that their preparations were contaminated with whole cells. At this time, there had been encouraging results obtained with extracts of Clostridium pasteurianum by Hock and Westlake (17). However, it was not until 1960 that consistent fixation was obtained with Cl. pasteurianum (9).

In the study of nitrogen fixation, there has been a variety of analytical methods used. The largest amount of usable data has been provided by the use of ^{15}N. There are several other methods that are used even though they are less sensitive. These include NH_3 distillation from the reaction mixture (24), ninhydrin detection

of amino acids formed (19), use of ^{13}N (27), and acetylene reduction (16).

There is considerable evidence indicating that NH_4^+ is an intermediate in nitrogen fixation. It has been (1)(8) demonstrated that NH_4^+ was one of the first products of the fixation process. Using whole cells of A. vinelandii, Burris added ^{15}N labeled nitrogen gas to a culture of the organism and then analyzed the cells for the labeled compounds. He found that the highest amount of label was in NH_4^+, the next highest in glutamic acid and a lesser amount in aspartic acid. These two amino acids contained very little in comparison to the amount of ^{15}N found in ammonia. Burma and Burris (6) showed the same sequence of labeling when $^{15}NH_4^+$ was offered to azotobacter. Mortenson (25) placed an atmosphere of labeled N_2 over cell-free extracts of Cl. pasteurianum. After the enzyme reaction had run for a length of time he removed the nitrogen gas and added alpha keto glutarate and demonstrated a decrease in the alkali distillable ^{15}N containing fraction of the preparation. He distilled over only the NH_3 leaving any nitrogen as amides that were formed to be included in the Kjeldahl nitrogen determination. Accompanying this decrease in labeled distillable nitrogen he also observed an increase in labeled glutamate indicating that glutamate dehydrogenase (GDH) was present.

In an attempt to determine where molybdenum is required in nitrogen fixation, Magee and Burris (21) looked for a molybdenum containing enzyme. Using cell-free extracts of A. vinelandii, one

of the enzymes they extracted was glutamate dehydrogenase. The authors used a crude extract to demonstrate the reduction of NAD as proof of the presence of glutamate dehydrogenase. The coenzyme NAD is required by this reaction in which glutamate is the substrate. The enzyme did not contain molybdenum. They demonstrated the presence of the enzyme GDH in cells grown on N_2.

Yalovleva et al. attempted to demonstrate GDH in Azotobacter vinelandii (34). They tried to use the reaction involving oxidation of $NADH+H^+$ which can be measured using the spectrophotometer. In this reaction NADH+H is the coenzyme, alpha-keto glutarate is the substrate, and NH_4^+ is also required. They found that an active $NADH+H^+$ oxidase system prevented use of the spectrophotometer as a means of following the reaction. The authors also used glutamate production to follow the reaction. Glutamate is an end-production of this reaction. Their data were meager from the measurement of glutamate. They concluded that the oxidation of $NADH+H^+$ could not be used as a method for measuring GDH activity of a crude extract. They did attempt one more step in purification, centrifugation of the crude extract at 105,000 xG for three hours. They demonstrated that the activity was precipitated with the particulate fraction of the extract.

Glutamate dehydrogenase has been purified from Azotobacter chrococcum which had been grown on N_2 (14). The authors determined the optimum pH, and the Km for the reaction utilizing NAD and glutamate.

The enzyme GDH has been demonstrated from a variety of different sources. In animals, it has been shown in beef liver (28), chicken liver (32), amphibian liver (2) and others. In plants the enzyme has been demonstrated in corn leaves (4) and wheat (18). All these different sources of the enzyme have one thing in common, that is the specificity of the enzyme for NAD. In microorganisms there are those that have a specificity for NAD and those with a specificity for NADP. Glutamate dehydrogenase from Escherichia coli (15) and Pasteurella tularensis (29) has been shown to be NADP specific. The enzyme in Neurospora crassa (31) and Thiobacillus novellus (20) has been shown to contain two different glutamate dehydrogenases, one is specific for NAD and the other requires NADP. LeJohn and McCrea (20) concluded that the NAD specific enzyme was prevalent during autotrophic growth and the NADP specific enzyme was more prevalent during hetrotrophic growth. In Azotobacter the enzyme is NAD specific (14)(34).

MATERIALS AND METHODS

Organism

The organism used in this study was Azotobacter vinelandii strain O obtained from the departmental culture collection. It was maintained on Burk's agar slants.

Medium

Burk's nitrogen-free medium (33) with the following modifications was used to grow the organism. Sodium citrate was added (0.8 gm per liter) to prevent precipitation of the calcium phosphate salts. This eliminates the necessity of autoclaving the phosphate salts separately. Ferric citrate (1 mg per liter) replaced the ferric chloride as the iron source. This medium was supplemented with the different nitrogen sources for growth of the organisms on them. The different nitrogen sources used were ammonium acetate, 1.38 gm per liter, D(+) glutamic acid, 4.5 gm per liter, and DL-aspartic acid, 5.0 gm per liter and atmospheric nitrogen.

Inoculum

Inoculum was prepared by the following procedure. A loop of the organism from the slant was transferred to a 500 ml erlenmeyer flask containing 150 ml of the nitrogen-free medium. This was placed on a rotary shaker for 18 hours.

Mass-culture

The cells were grown in 500 ml flasks containing 150 ml of Burk's medium and 4 ml of inoculum. These flasks were placed in a rotary shaker to obtain maximum areation. When the other nitrogen sources were used, a New Brunswick Micro-ferm fermentor model MF-107 was used. Six liters of sterilized Burk's medium with the appropriate nitrogen source was added to the sterile fermentor. The fermentor was inoculated with 300 ml of liquid culture prepared as described above. The atmosphere used with the fermentor was a 1:5 mixture of oxygen and helium. This atmosphere prevented the use of atmospheric nitrogen as a nitrogen source by the organisms. The cells were harvested during the early exponential phase of growth.

Harvest of cells and preparation of cell-free extracts

Cells were harvested with a Szent-Gyorgi and Blum KSB 8 tube continuous system on a type SS-3 Servall centrifuge at 9700 x G. After harvesting, the cells were washed three times by resuspending them in 0.2M K_2HPO_4-HCl buffer pH 7.8 and recentrifuging them from it. This buffer was used throughout the experiments. A Model PR-2 International refrigerated centrifuge with a multiple speed attachment at -5° C was used at 20,000 x G to precipitate the cells. Washing of the cells was done three times.

The pellet of cells was weighed and then resuspended in the 0.2M buffer at a concentration of 1:2 (w/v). This suspension was then poured into a cold French pressure cell at 4 C for breakage.

The pressure was brought to 10,000 psi and the cells were allowed to equilibrate for fifteen minutes before being released from the pressure cell. The whole cells and other debris were removed using the Model PR-2 International centrifuge at 20,000 x G for fifteen minutes. The resulting supernatant liquid was labeled Fraction A. This fraction was dialyzed for four hours against one liter of 0.2 M phosphate buffer at 4 C. The activity was determined as described below and a portion of this fraction was further purified by centrifugation with a Model L Spinco ultracentrifuge for three hours at 122,000 x G using a type 50 head. The supernatant fluid containing the enzyme was labeled Fraction B.

The activity of each fraction was measured by following the reduction of nicotinamide-adenine dinucleotide (NAD)(Sigma Chemical Co.) at 340 nm using a Beckman DU spectrophotometer. The test mixture contained: 50 µmoles of D (+) glutamic acid, 9 µmoles of NAD and from 25 to 50 mg of protein per ml as cell extract. The remaining three ml in the pyrex reaction cuvette was made up of 0.2 M phosphate buffer. All reagents were made up in the same phosphate buffer described above. Each reaction was started by the addition of the glutamate.

The concentration of protein was determined by spectrophotometric readings at 280 nm and 260 nm (13).

End-product detection

The method used for the extraction of alpha-keto glutarate (AKG) was a combination of parts of the methods of Cavallini et al.

(10) and Metzler and Snell (22). Three volumes of 5 percent metaphosphoric acid were added to 1 volume of reaction mixture to precipitate the protein. The precipitated proteins were removed by centrifugation at 17,000 x G for fifteen minutes with the Servall SS-3 centrifuge. The supernatant fluid from each centrifuge tube was decanted into a ten ml serum bottle and 1.5 ml of 0.2 percent 2,4-dinitrophenylhydrazine in two N HCl was added. This mixture was incubated at 37 C for 20 minutes. The material was then transferred to a separatory funnel and extracted four times with ethyl acetate with mixing by bubbling N_2 through it. The ethyl acetate was saved each time. This was then extracted four times with a two ml aliquot of 10 percent sodium carbonate. The aqueous sodium carbonate extract was saved each time. These were combined and extracted with two ml of ethyl acetate. The aqueous layer containing the alpha-keto glutarate as its sodium salt was saved (10). Five ml of 2.5 N sodium hydroxide was added to five ml of the aqueous extract and allowed to stand for ten minutes. The optical density was read at 520 nm using a Bausch and Lomb Spectronic 20 Spectrophotometer (22).

The quantity of alpha-keto glutarate was determined using the same reaction mixture and cell-free extract as described above for NAD reduction. The reactions were stopped at given time intervals by the addition of nine ml of 5 percent metaphosphoric acid. In the 0 time sample the acid was added before the addition of the enzyme to prevent any possible production of AKG.

Standard curve of reduced NAD

The standard curve for reduced NAD was determined by preparing solutions of NAD ranging in concentrations from 1.8 to 9.0 μmoles per 0.5 ml. Each concentration was placed in a three ml Pyrex spectrophotometer cuvette. The phosphate buffer previously described was added to bring the total volume in each cuvette to three ml. The reduction was accomplished by adding 0.2 ml of 0.1 N sodium hydrosulfite (13). The optical density was read at 340 nm before the addition of the sodium hydrosulfite and after the reduction had been completed. The optical density readings at the different concentrations were plotted against the umoles of NAD added to each solution. The optical density from this graph was used to convert the optical density resulting from the reduction of NAD during the course of the enzyme reactions to umoles of NAD reduced.

RESULTS AND DISCUSSION

In order to find a rapid and simple method for the detection of glutamate dehydrogenase (GDH), several methods of assay were tried. One method that held much promise was gel electrophoresis (12). When a protein dye, comasse blue, was used to stain the bands after separation of the material on the gels, there were so many bands that it was difficult to determine where one band stopped and the next one started. In order to determine where the enzyme was located after migration of the material, the gels were placed in a solution containing nicotinamide-adenine dinucleotide (NAD), glutamic acid, phenazine methosulfate (PMS) and oxidized nitroblue tetrazolium (NBT). Enzyme activity is detected when during the reaction electrons are passed to NAD. The reduced NAD is auto-oxidized by PMS which in turn passes the electrons to the NBT which turns blue upon reduction. There were a large number of blue bands observed indicating that there were other reactions going on that would transfer electrons to the NBT. Contamination of the GDH with other systems capable of reducing the NBT renders this method useless.

Sephadex G-200 was used as a molecular sieve by Roger and his co-workers (30) to determine the molecular weight of L-glutamate dehydrogenase. Even though these authors used purified GDH in their experiments, it was thought that this method could be used to remove some of the unwanted material such as nucleic acid and

the lower molecular weight proteins which would be trapped by the dextrans. The capacity or bed volume of the column dictates how much protein can be placed on it so as not to overload it. In order to obtain a sufficient quantity of enzyme for assay it would have been necessary to overload the column to a point of plugging it up; therefore, this method could not be used.

GDH activity is shown from extracts of cells grown on several nitrogen sources using centrifugation methods described in Materials and Methods.

The umoles of NAD were read from the standard curve (Figure I). Figure II shows the activity obtained from preparations grown on N_2 and ammonium acetate. The graph labeled N_2 shows the result obtained when cells were grown using atmospheric nitrogen as their nitrogen source. Line A indicates the activity of Fraction A and line B is the activity obtained from the ultracentrifugation of Fraction A. The graph labeled NH_3 on Figure II shows GDH activity of preparations from cells grown with ammonium acetate as their nitrogen source. The activity of Fraction A is represented in Line A, and the activity obtained from the supernatant liquid from the ultracentrifugation of Fraction A is represented by Line B. In comparing the two A fractions from the two graphs very little difference is noted in the slope of the lines. Glutamate dehydrogenase appears to be present in A. vinelandii in almost equal amounts whether the cells are grown on N_2 or NH_4^+ as their nitrogen source. The specific activity of each fraction from each nitrogen source is compared in Table I.

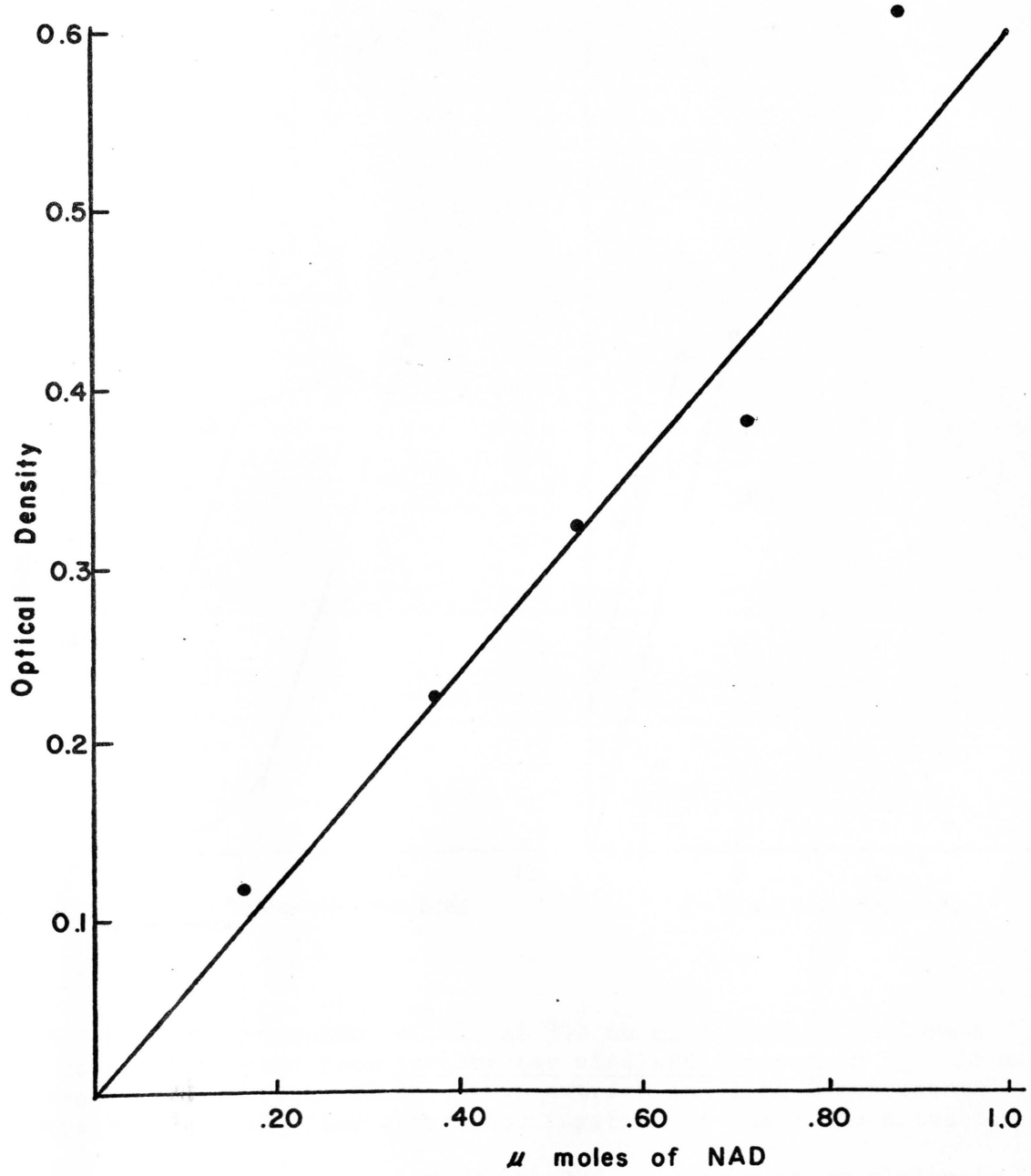

Figure I

Standard curve from chemical reduction of NAD. The optical density was read at 340 nm after the reduction of each concentration of NAD has been completely reduced by sodium hydrosulfite.

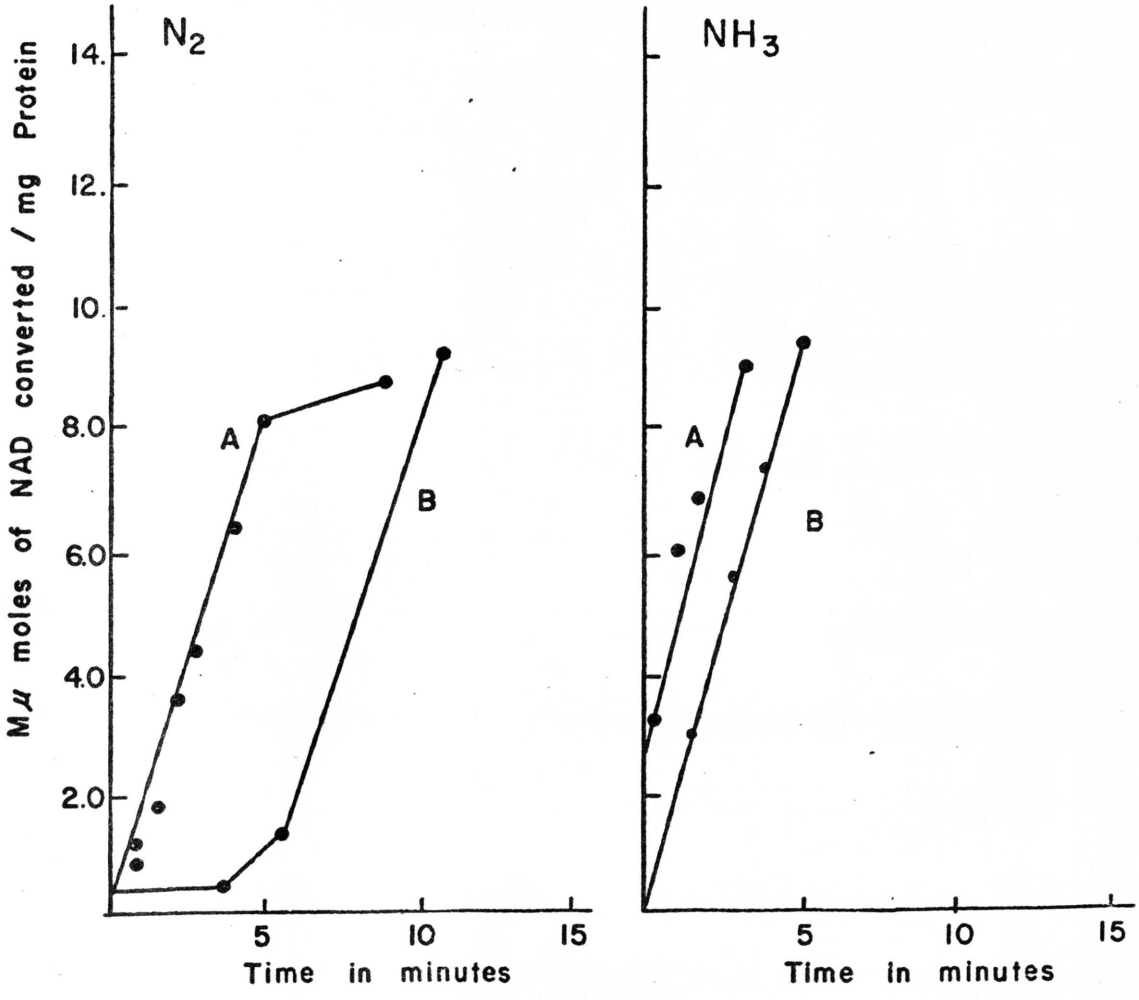

Figure II

Graph N_2 shows the reduction of NAD at 340 nm catalyzed by glutamate dehydrogenase extracted from Azotobacter vinelandii grown on N_2. Line A represents the activity of the crude extract and line B represents the activity obtained after ultracentrifugation of the crude extract.

Graph NH_3 shows the reduction of NAD at 340 nm catalyzed by glutamate dehydrogenase extracted from Azotobacter vinelandii grown on ammonium acetate. Line A represents the activity of crude extract and line B represents the activity obtained after ultracentrifugation of the crude extract.

Table 1. Specific Activity of Glutamate Dehydrogenase from Extracts of Azotobacter Vinelandii

Cells grown on	Enzyme fraction	Mμmoles activity*	Percent increase in activity
N_2	A	1.57	5
	B	1.65	
Ammonium acetate	A	1.2	100
	B	2.3	
Glutamate	A	0.56	480
	B	2.7	
Aspartate	A	.035	210
	B	.78	

*Mu mole of NAD converted/min./mg protein

In comparing the specific activity of the A fractions, it can be seen that the specific activity of cells grown on glutamate and aspartate were considerable lower than the activity obtained from cells grown on N_2 and ammonium acetate. This difference in the amount of GDH in the cells grown on N_2 and ammonium acetate compared with those grown on amino acids may indicate that more of the enzyme was extracted from the cells. The trend toward increased activity in Fraction B over Fraction A is significantly greater in the extracts from cells grown on the amino acids over extracts from the other two nitrogen sources.

Figure III shows GDH activity of preparations from azotobacter grown with amino acids as their source of nitrogen. The Fraction A activity is considerable lower than that from cells grown on N_2 or NH_4^+. The activity of Fraction B was a good deal higher.

The production of AKG (Figure IV) was determined using Fraction A as the enzyme source. The reaction mixture contained the same quantities of NAD, glutamate and phosphate buffer as were used in measuring the reduction of NAD with the spectrophotometer. Each reaction was studied to show the increase in the product with time. The procedure of extraction of the AKG has been described. Figure IV shows that there was an increase in the amount of AKG produced with time. The graph shows that less than one tenth of the glutamate was converted to AKG during the course of the reaction. It was not the object of this experiment to compare activity of the different fractions only to show that AKG was being produced and to give some indication of how much. The fact that only about one-tenth of the glutamate was used indicates that it was not a limiting factor in the reactions.

The fact that the enzyme was found in the supernatant fluid by this experimenter is contrary to what was found by Yakovleva and his co-workers (34). They found the activity remained in the particulate fraction of a ultracentrifugation at 105,000 x G for three hours. The reason for this difference may be explained by the method used to break the cells. This author used the French pressure cell and Yakovleva used the sonicator. The activity was

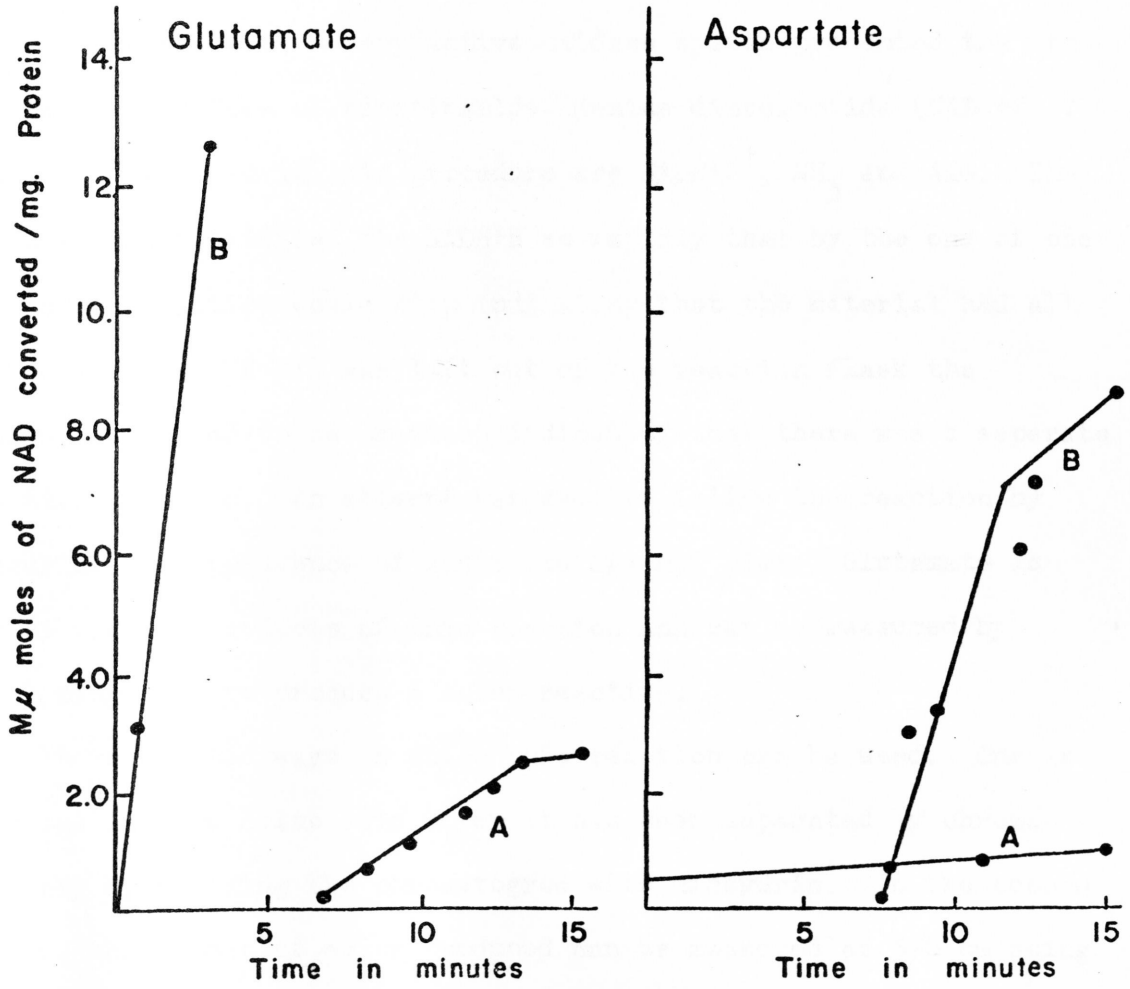

Figure III

Graph glutamate shows the reduction of NAD at 340 nm catalyzed by glutamate dehydrogenase extracted from Azotobacter vinelandii grown on glutamate. Line A represents the activity of crude and line B represents the activity after centrifugation of the crude extract.

Graph aspartate shows the reduction of NAD at 340 nm catalyzed by glutamate dehydrogenase extracted from Azotobacter vinelandii grown on aspartate. Line A represents the activity of the crude extract and line B represents the activity obtained after ultracentrifugation of the crude extract.

never found in the particulate fraction after ultracentrifugation by this worker.

The presence of a very active oxidase system prevented the use of the reduced form of nicotinamide-adenine dinucleotide ($NADH+H^+$). The reactants used in this procedure are $NADH+H^+$, NH_3 and AKG. The oxidase system oxidized the NADH+H so rapidly that by the end of one minute the reaction would stop indicating that the material had all been oxidized. If AKG was left out of the reaction flask the $NADH+H^+$ continued to be oxidized indicating that there was a separate reaction going on. An attempt was made to follow the reaction by measuring the appearance of glutamate against time. Glutamate is one of the end-products of this reaction and can be measured by using ninhydrin to produce a color reaction.

There are two ways in which this reaction can be used. One is to visualize the amino acid after it has been separated by chromatography by spraying the chromatogram with ninhydrin. In the second method, the amount of color produced can be measured at 570 nm using the Bausch and Lomb Spectronic 20 spectrophotometer (23). Neither method was applicable to this study. Thin-layer chromatography could not be used to obtain quanitative data on the end-product. The presence of interfering materials in the reaction mixture made the colorimetric method unusable. The presence of an $NADH+H^+$ oxidase system prevented Yakovleva et al. (34) from obtaining significant results when they used the reaction mixture containing $NADH+H^+$, NH_3, and AKG. They also attempted to use glutamate

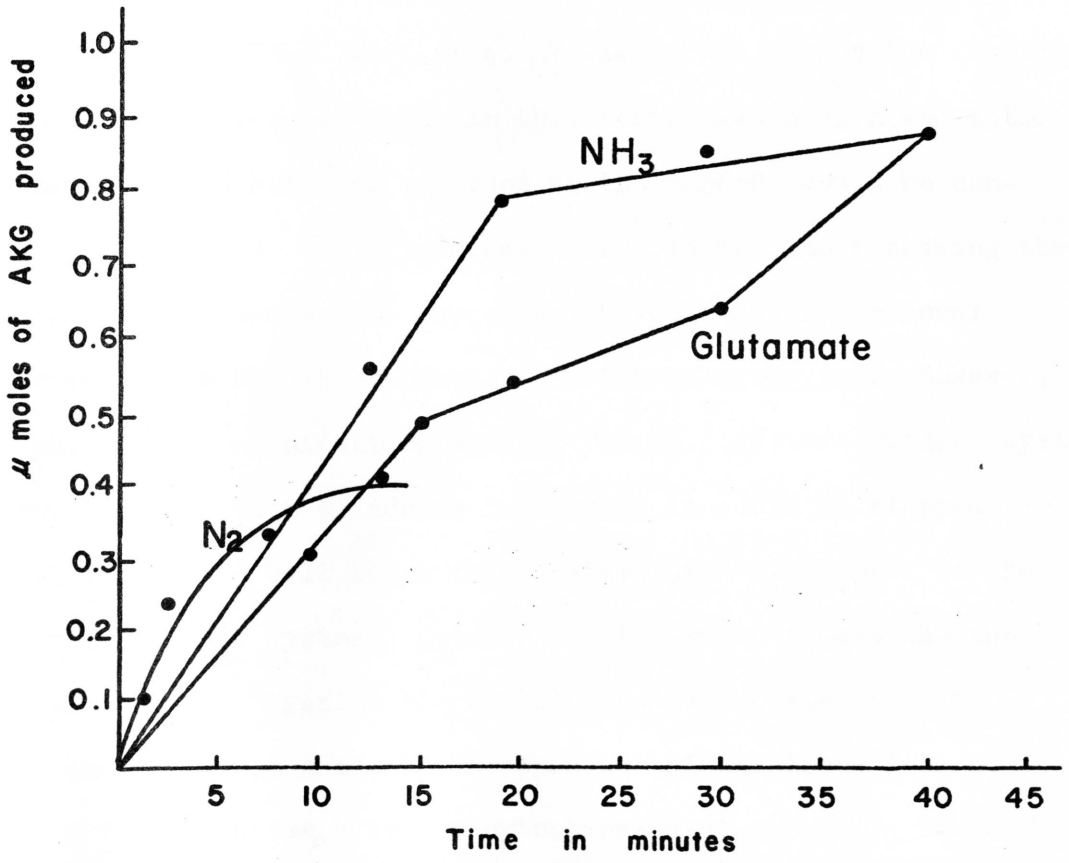

Figure IV

Results obtained when umoles of alpha-keto glutamate produced was plotted against time. The crude extract from the different nitrogen sources was used for the enzyme source. Line N_2 represents production of AKG using crude extracts from cells grown on N_2. Line NH_4^+ represents production of AKG using crude extract from cells grown on ammonium acetate. Line glutamate represents production of AKG using crude extract from cells grown on glutamate.

production as a measure of activity and had only limited success.

There was a consistent lag period observed before NAD reduction started. This lag was believed to have been caused by the above described oxidase system. It was thought that this system was reoxidizing the $NADH+H^+$ as fast as it was formed during the reaction. The terminal electron acceptor in this system was oxygen and until all the oxygen in solution was used up the $NADH+H^+$ would be continually reoxidized. This idea was disproved by simply shaking the cuvette to incorporate more oxygen which would allow continual oxidation of the NADH+H and extension of the lag period. However, this shaking had no effect on the lag period. If this oxidase system was functioning, then by adding inhibitors it could be stopped. Several inhibitors will block this system which is coupled to the cytochrome oxidase system. Cyanide and Antimycin A were the two inhibitors tried. Cyanide blocks the transfer of electrons to oxygen and Antimycin A blocks the transfer of electrons from coenzyme Q to cytochrome b in the cytochrome system. (11) These inhibitors had no effect on the lag period. The reason for the lag is still unknown. This is an area that needs further work.

The appearance of aspartate as the amino acid with the second highest label, second only to glutamate, led to the following experiment. The method of Halpern and Umbarger (15) was used to determine the presence of an active aspartase. The preliminary data indicated that aspartase was not present. This is another area where more work could be done. It would be interesting to

determine if the NH_3 produced from the action of nitrogenase is used to form glutamate and the amino group transaminated to oxalacetate to form aspartate or whether it is formed directly by amination of oxalacetate.

CONCLUSIONS

1. Several methods of isolation were used in an attempt to demonstrate glutamate dehydrogenase from Azotobacter vinelandii, only centrifugation proved to be effective.
2. The enzyme was shown to be present in A. vinelandii cells when they were grown using either N_2, ammonium acetate, glutamate, or aspartate as the sole source of nitrogen.
3. Glutamate dehydrogenase is apparently not involved.
4. An attempt to demonstrate aspartase in A. vinelandii was unsuccessful.

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